Riboflavin-mediated extracellular electron transfer process involving *Pachysolen tannophilus*

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A B S T R A C T

Microbes can generate electricity in microbial fuel cells and transform contaminants in nature, utilizing extracellular electron transfer (EET). Fungi exist widely in environment but few studies have concerned an associated EET mechanism and so their application is limited. In this study electrochemical techniques were employed to study the extracellular reduction capability of fungi using a representative model *viz.* *Pachysolen tannophilus*; a solid electrode and soluble Cr(VI) were used as extracellular electron acceptors. Despite a thick cell wall, the yeast was proved to be electrochemically active and an obvious redox peak was observed at -0.41 V in differential pulse voltammetry. Analysis of electrochemical and HPLC data led to the proposal of an EET with riboflavin as the electron transfer mediator. Riboflavin secreted by the yeast was also responsible for the transformation of Cr(VI) to less mobile and less toxic Cr(III). Further results demonstrated that the acidophilic *P. tannophilus* secreted riboflavin to a concentration of 0.26 μmol/g biomass at pH 3, but only to 0.15-0.16 μmol/g biomass at pHs between 4 and 7. The findings contribute to the understanding of biogeochemical processes and further contribute to innovative remediation of polluted environments.

1. Introduction

Electron exchange between microbes and chemicals is crucial for microbial fuel cells (MFCs) and for biogeochemical cycling [1]. MFCs transform chemical energy into electrical energy via electrochemical reactions involving extracellular electron transfer (EET) with microbes. The EET enables organisms to use extracellular electron acceptors for cell metabolism [2], *i.e.* electron acceptors bound to and distant from organisms are able to be transformed through EET pathways. Microbial EET via proteins or via electrochemically active metabolites has been observed. Mediated EET predominates in the interaction between microbes and extracellular electron acceptors without direct cell contact [3,4]; the types of mediators present in the environment play a vital role in biogeochemical cycling processes via such EET [5].

Riboflavin, a redox active compound, is secreted by many microbes [6,7]. In some MFCs, riboflavin-based EET has been reported to dominate electrical current production [3]. Studies of the EET mechanism have mainly concerned Gram-negative bacteria *e.g. Shewanella* or *Geobacter* [8]. Fungi exist widely in environment and have the biochemical and ecological capacity of organic compounds degradation and affecting the fate of metals in biogeochemical cycling [9]. In contrast to Gram-negative bacteria, fungi are enveloped by a thick (100–200 nm) cell wall [10] which could change the EET pathways. Rousk et al. found that the biomass of fungi increased with a lowering of soil pH, whereas the biomass of bacteria decreased [11]. Therefore, research that focuses on the EET mechanism involving fungi will aid the understanding of the diversity of biogeochemical processes in acidic environments and will further promote the application for environmental remediation by MFCs. In the case of fungi, only a few yeast-based MFCs have been reported; those could be used for simultaneous electricity generation and phytate bioremediation [12,13], but the EET process with those yeasts remains unclear [14].

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Biogeochemistry is dominated by redox reactions initiated between cells and the environment [15]. Bioelectrochemical techniques that have been widely used in MFCs study are able to easily characterize redox reactions that happen during cell EET [16]. Such bioelectrochemical techniques can therefore provide important information about biogeochemical processes in nature [1]. *Pachysolen tannophilus* is a model yeast that has been extensively studied in biological sciences. In this study bioelectrochemical techniques have been used to study the EET mechanism for *P. tannophilus*. The role of riboflavin secreted by the yeast on EET has been explored with a solid electrode and with soluble Cr(VI) as electron acceptors. The effect of different pHs on the efficiency of riboflavin secretion has also been studied.

2. Materials and methods

2.1. Microbial cultures

P. tannophilus (CGMCC 2.1622) was purchased from the China General Microbiological Culture Collection Center. Flavins are reported to be contained in yeast extract [17]. Hence, instead of yeast peptone dextrose (YPD) medium a yeast-extract-free YPD(Y-) medium (*i.e.* 20 g L^{-1} peptone and 20 g L^{-1} glucose) was prepared for the cultivation of *P. tannophilus*. To characterize the riboflavin secreted at different pHs (*i.e.* 1.8, 2.0, 3.0, 4.0, 5.8, 7.0), the pHs were adjusted by 1 M NaOH or 3 M HCl [18]. An inoculation ratio of 1% was applied in liquid culture. The *P. tannophilus* cells were incubated in a shaker (32 °C, 150 rpm).

2.2. Electrochemical measurements

Electrochemical experiments were conducted using a CHI660D electrochemical workstation and a CHI1000 B potentiostat (CH Instruments, China). For cyclic voltammetry (CV) and differential pulse voltammetry (DPV), a 3 mm diameter glassy carbon electrode, a platinum wire and a saturated Ag/AgCl reference electrode (+197 mV vs. standard hydrogen electrode) were used as the working electrode, counter electrode and reference electrode, respectively. In this article, all potentials are reported versus the saturated Ag/AgCl reference electrode. The electrolyte used was a phosphate buffer solution (PBS, 50 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH = 7.0). All the solutions used in the electrochemical experiments were N$_2$(g) purged to guarantee that oxygen was eliminated.

To detect redox active compounds in the culture, the supernatant was harvested and adjusted to pH 7.0, and then used for CV and DPV tests. Riboflavin that dissolved in PBS to a final concentration of 1.5 μM was also used for CV and DPV tests. For characterization of the electrochemical response of *P. tannophilus*, the stationary phase cells were harvested and washed with PBS. A 5 μL cell aggregate was transferred onto the glassy carbon electrode and coated with 5 μL Nafion ionomer (5% dispersion), then dried at room temperature. The glassy carbon electrode with bound *P. tannophilus* was used for CV and DPV tests [19]. In CV experiments, the potentials range was −0.6 to 0.4 V and the scan rate was 10 mV s^{-1}. In DPV experiments, the range was −0.6 to −0.2 V, the staircase was 0.004 V, the pulse width was 0.05 s, the pulse period was 0.2 s (*i.e.* scan rate = 20 mV s^{-1}).

A three-electrode configuration was used in chronoamperometry. Carbon felts of 9 cm^2 served as the working and counter electrodes. A saturated Ag/AgCl electrode was employed as reference. These electrodes were put in a 100 mL bottle sealed by a butyl rubber plug to sustain an anaerobic environment. YPD(Y-) medium amended with 50 mM PBS served as the electrolyte (pH = 7).

For identification of electrochemical activity of *P. tannophilus*, the three-electrode cell was inoculated with 1% (v/v) mid-log phase *P. tannophilus* (OD$_{600}$ = 5 or 1.79 g dry weight biomass per liter), and a *P. tannophilus*-free three-electrode cell was used as the control. The potential of the working electrode was held at +0.40 V in the chronoamperometric experiments to identify electrochemical activity of *P. tannophilus*.

To characterize the role of riboflavin in EET, the potential of the working electrode was poised at −0.30 V for three groups: 1) 1% (v/v) of *P. tannophilus* inoculant and riboflavin (final concentration = 10 μM) were added to the three-electrode cell; 2) only *P. tannophilus* (1%, v/v) was inoculated; 3) abiotic control experiment, i.e. systems with final concentration 10 μM of riboflavin and without *P. tannophilus*. All tests were carried out in triplicate.

2.3. Chemical analysis

HPLC was employed to determine riboflavin content. *P. tannophilus* was incubated in YPD(Y-) medium. The cell suspension was centrifuged (7000 g, 10 minutes) to separate cells from the supernatant. The cell pellet was then dried and the weight was recorded. The supernatant was filtered through a 0.22 μm filter. A HPLC instrument equipped with a reversed-phase C18 column (LC-1200, Agilent, USA) was used for the analysis of riboflavin [20]. The mobile phase was methanol (89%), 650 mM acetic acid (1%) and deionized water (10%) at a flow rate of 1 mL min^{-1}. Riboflavin was determined using an Agilent 1200 FLD fluorescence detector with an excitation wavelength of 450 nm and an emission wavelength of 516 nm. A calibration curve was established, linking riboflavin concentration and peak area. Riboflavin concentration was calculated by the area of the peak at a retention time of 8.75 min. The HPLC data of riboflavin, *P. tannophilus* culture supernatant and YPD(Y-) medium are shown in Fig. S1.

For Cr(VI) reduction, *P. tannophilus* cells were collected at the stationary phase by centrifugation (7000 g, 10 minutes). All the solutions were purged with N$_2$ gas (purity ≥ 99.999%); the suspension was incubated in a serum bottle that was sealed with a butyl rubber plug to sustain an anaerobic environment. To minimize the effect of Cr(VI) toxicity on the growth of the cells, the collected cells were re-suspended in fresh YPD(Y-) medium to a high density (2.70 ± 0.01 g dry weight per liter or OD$_{600}$ = 6.18 ± 0.02) and then Cr(VI) was added to a low concentration of 10 mg L^{-1} [21]. Both Cr(VI) and riboflavin (final concentration = 10 μM) were added to the serum bottle for the *P. tannophilus* + Riboflavin, whereas only Cr(VI) was added for the *P. tannophilus*. In addition, abiotic experiments (cell-free, the Control and the Riboflavin) were also performed. Cr(VI) concentration was determined by the standard diphenylcarbazide method using a UV-1200 spectrophotometer (Shanghai, China). Total Cr was determined using a M6 flame atomic absorption spectrometer (Thermo, USA) as in previous work [22].

3. Results and discussion

3.1. Detection of riboflavin secreted by tannophilus

Flavins are yellow chromophores that exist in the form of riboflavin, flavin mononucleotide and flavin adenine dinucleotide. Most yeasts have a riboflavin excretion system [6,23]. We therefore detected whether there was any riboflavin in the supernatant of culture using HPLC. Fig. 1a shows that riboflavin content increased concomitantly with the growth of *P. tannophilus*. Glucose strongly activates the riboflavin excretion process [6]. After cell growth reached the stationary phase, riboflavin concentration increased slightly and fluctuated around 0.45 μM.

To demonstrate the involvement of an EET pathway, cyclic voltammetry experiments were performed in the cell-free supernatant with a glassy carbon electrode. In Fig. 1b, the CV
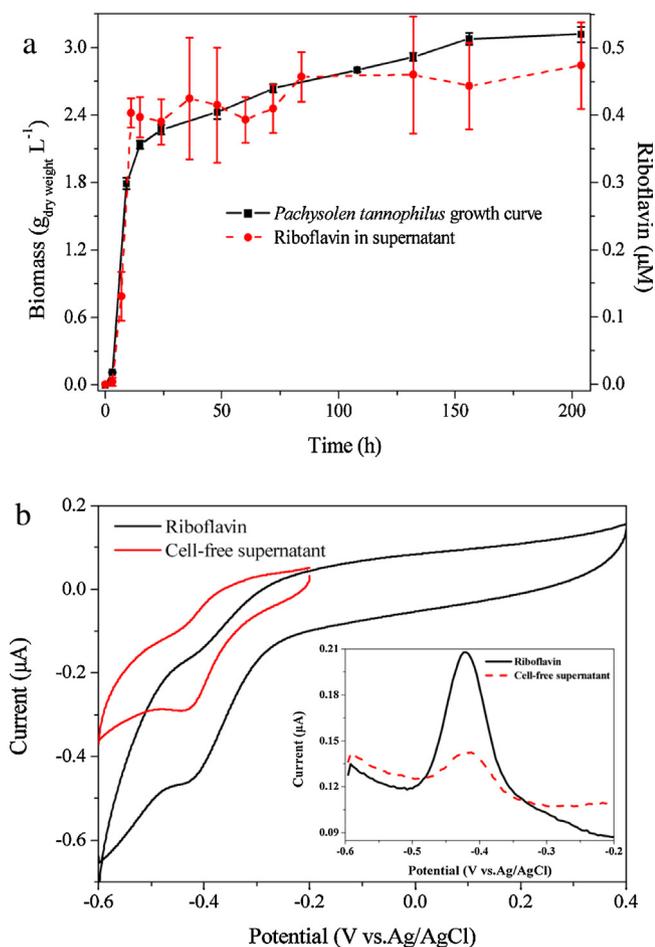
Fig. 1. (a) The growth curve (solid line) of *P. tannophilus* and the riboflavin concentration in the corresponding supernatant (dashed line). (b) CV and DPV (insert) of 1.5 μM riboflavin (solid line), cell-free culture supernatant (dashed line).

Fig. 2. (a) CV trace of bare glassy carbon working electrode (solid line) and of *P. tannophilus* (dashed line) on a glassy carbon working electrode. (b) Chronoamperometry in the absence (solid line) and presence (dashed line) of *P. tannophilus*. The potential of the working electrode was held at +0.4 V.

traces of the riboflavin solution and the cell-free supernatant showed redox peaks around −0.40 V. By minimizing the capacitive current, DPV can detect lower redox signals than the CV technique [16]. DPV of the cell-free supernatant showed an obvious redox peak at −0.41 V, which was the same as that of riboflavin solution (Fig. 1b insert). In the light of the oxidation current produced by the supplemented riboflavin at 1.5 μM, the concentration in the cell-free supernatant was calculated to be *ca.* 0.44 μM riboflavin, close to the result from HPLC (0.45 μM). Hence, DPV provided a mean to characterize the concentration of redox active secretions, especially the secretions with low concentration. Results in Fig. 1 demonstrated that *P. tannophilus* was able to secrete redox active riboflavin to the culture.

3.2. Characterization of the electrochemical activity of the yeast

In Fig. 2a, the CV trace of *P. tannophilus* shows two weak redox peaks around −0.40 V and two pronounced redox peaks at 0.11 V and −0.17 V. *Shewanella oneidensis* has been found to have a similar redox response in CV traces [24,25]. DPV of *P. tannophilus* was conducted to further characterize the oxidation peak around −0.40 V. It was noted that riboflavin, cell-free supernatant and *P. tannophilus* all had the same oxidation peak potentials at −0.41 V in DPV traces (Fig. S2). Combining the electrochemical results with the HPLC data (2a), we conclude that *P. tannophilus* cells secrete riboflavin to the culture and that the redox peaks at approximately −0.40 V are due to riboflavin.
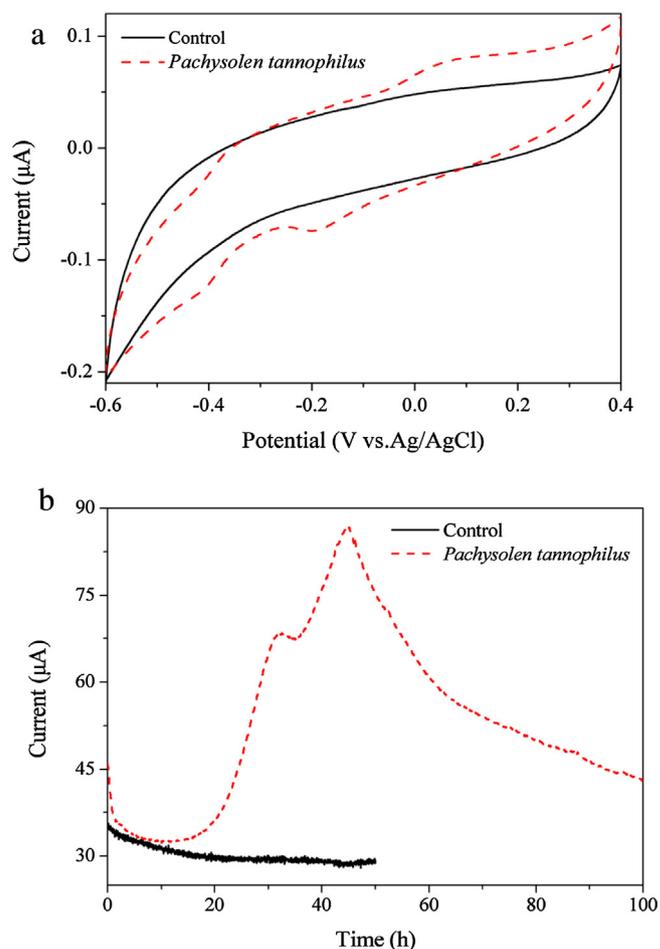
In our preliminary experiment, the data indicated that the addition of glucose aroused the electrocatalytic current when compared the turnover CV with the non-turnover CV (Fig. S3). Chronoamperometry technique was employed to further identify the electrochemical activity. When working electrodes are set at the redox potentials of electron acceptors in the natural environment, the redox reaction on the electrode surface is able to mimic biogeochemical processes [26]. In this study, chronoamperometry was used to profile electron transfer between microbes and a solid substance as a function of time [2,26]. In Fig. 2b no current change as a function of time was found in the abiotic control, while the current increased when *P. tannophilus* was inoculated into the three-electrode setup. The peak current of 84.8 ± 12.8 μA indicated that *P. tannophilus* can transfer electrons from cells to a solid electrode.

Previous reports demonstrated that yeast can generate electricity in MFCs [27], but few reports focused on the associated EET mechanism [28,29]. Uric acid and some possible trans-plasma membrane transport proteins were proposed to be responsible for EET of yeast [14,30]. However, uric acid only showed an oxidation peak in CV, and it is different from that of riboflavin in this work. Even though there is a thick cell wall, our CV and chronoamperometry results indicate that *P. tannophilus* has a good EET capacity, which may be via some redox active electron transfer mediator, *i.e.* via riboflavin.

3.3. Effect of riboflavin on EET

The working electrode is able to serve as a solid electron acceptor for cell metabolism when it is held at a suitable potential. To evaluate the role of riboflavin on EET of *P. tannophilus*, the working electrode was held at −0.30 V based on the redox peaks in CV trace. As presented in Fig. 3a, when riboflavin was added into the three-electrode cell, the current increment (40.4 ± 4.1 μA) was higher than that without addition of riboflavin (8.8 ± 1.4 μA), which cells can secret riboflavin into solution. No current change as a function of time was observed in the abiotic control. We therefore concluded that *P. tannophilus* employs riboflavin to transfer electron for cell metabolism.

Chromium (Cr) has aroused anxiety concerning a series of environmental problems. The transformation of carcinogenic Cr (VI) to less mobile and less toxic Cr(III) can be crucial in remediating a contaminated site. In Fig. 3b, we also evaluated the role of riboflavin on *P. tannophilus* in reducing the soluble Cr(VI) electron acceptor. The Cr(VI) content was 10.0 mg L^{-1} at 0 h, which decreased to 8.16 mg L^{-1} by addition of medium. The reason for the difference is that some medium components (*e.g.* glucose) reduced part of the Cr(VI) immediately on its addition to the system. The Cr

(VI) concentration decreased to 4.60 mg L^{-1} at 0.1 h with riboflavin addition. Moreover the Cr(VI) concentration kept constant in the following 10 hours in the Riboflavin experiments (Fig. 3b, dash line). Xafenias et al. [31] found that the increase of riboflavin concentration promotes the Cr(VI) reduction under abiotic condition. So the fast reduction of Cr(VI) with riboflavin addition may be aroused by the electron donating capacity of reduced riboflavin. From 0.1 h to 2 h, the Cr(VI) concentration decreased from 8.17 mg L^{-1} to 7.06 mg L^{-1} in the *P. tannophilus* experiments (Fig. 3b, dot line), whereas the Cr(VI) concentration decreased from 4.60 mg L^{-1} to 0.89 mg L^{-1} in the *P. tannophilus* + Riboflavin experiments (Fig. 3b, dash dot line). The reduced Cr(VI) between 0.1 h and 2 h in the *P. tannophilus* + Riboflavin experiments was 2.3 times higher than that in the *P. tannophilus* experiments. These results indicated that riboflavin promotes the reduction of soluble electron acceptors [25].

Both of riboflavin and humic substances contain quinone/phenolic moieties, which can serve as electron shuttles [32]. The results in Fig. 3 demonstrate that riboflavin facilitates the EET from *P. tannophilus* to a solid electrode and to soluble Cr(VI). This study therefore contributes to the understanding of the crucial role of redox active moieties contained in humic substances in the transformation and speciation of contaminants in natural environment [5,33].

3.4. Riboflavin secretion in acid environments

Fungi play a key role in biogeochemical transformation of minerals, heavy metals and other contaminants in acid environments [34,35]. Hence, the riboflavin secreted at different pHs (*i.e.* 1.8, 2.0, 3.0, 4.0, 5.8, 7.0) was studied in this work (Fig. 4). As presented in Fig. 1a, *P. tannophilus* secreted riboflavin mainly at exponential stage. When *P. tannophilus* growth reached stationary stage, the riboflavin content in culture varied slightly. The stable concentration of riboflavin at the long stationary stage (*ca.* 190 h) indicated the lysis of cell contribute little to the content of riboflavin. *P. tannophilus* was therefore cultivated at different pHs for 6 days to guarantee that the growth of *P. tannophilus* reached stationary stage and the concentration change of riboflavin is negligible. In Fig. 4 the harvested biomass and the riboflavin content varied slightly (0.15-0.16 μmol/g biomass) when the medium's pH was above 4.0. Since *P. tannophilus* is an acidophilic microbe, the content of metabolite is high in weak acid environments [36]. When the pH decreased to 3.0, the harvested biomass became lower than that of pHs above 4.0, but the produced
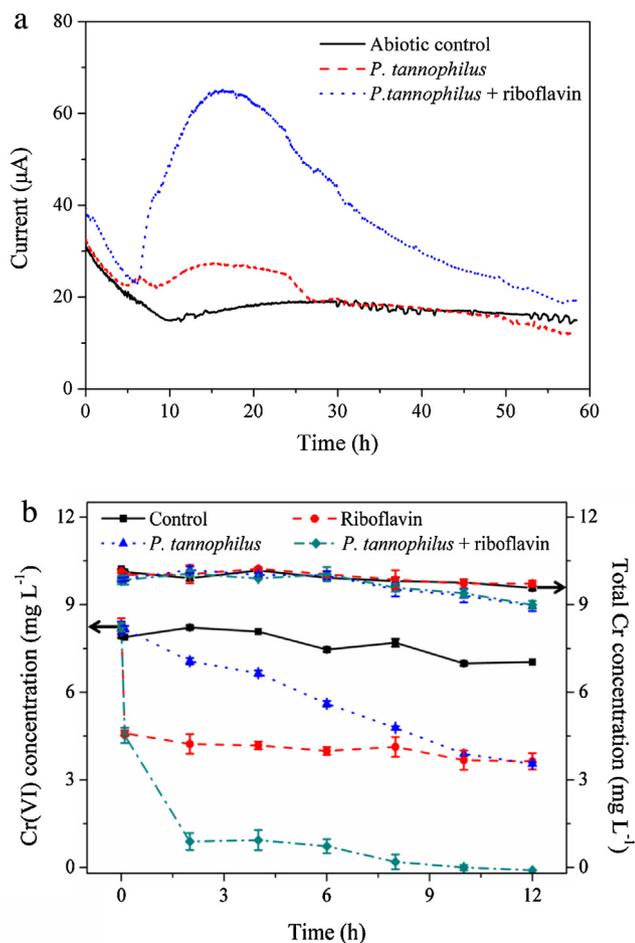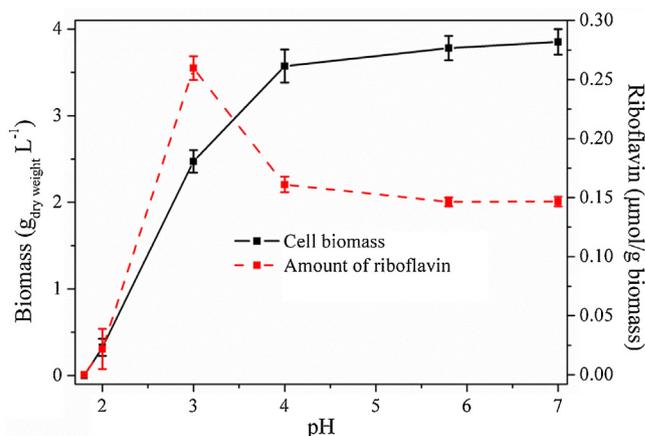
Fig. 3. (a) Chronoamperometry of *P. tannophilus* in the absence (dashed line) and presence (dotted line) of riboflavin. The abiotic control (solid line) was *P. tannophilus*–free medium with riboflavin. The potential of the working electrodes were held at −0.30 V. (b) *P. tannophilus* reduces soluble Cr(VI) in the suspension. The arrows indicate that the four lines depict the Cr(VI) concentrations and total Cr concentrations in the supernatants, respectively. Control: 10 mg L^{-1} Cr(VI) (square, solid line). Riboflavin: 10 mg L^{-1} Cr(VI) and 10 μM riboflavin (circle, dash line). *P. tannophilus*: 10 mg L^{-1} Cr(VI) and *P. tannophilus* (triangle, dot line). *P. tannophilus* + Riboflavin: 10 mg L^{-1} Cr(VI), 10 μM riboflavin and *P. tannophilus* (diamond, dash dot line).

Fig. 4. The biomass (solid line) of *P. tannophilus* and the amount of riboflavin (dashed line) in the corresponding supernatants after being cultured for 6 days at different pHs: 1.8, 2.0, 3.0, 4.0, 5.8, 7.0.

riboflavin content became higher to 0.26 μmol/g biomass. No obvious biomass or riboflavin content was detected when the pH was below 2.0.

These results indicate *P. tannophilus* secretes riboflavin in a weakly acidic environments; the riboflavin present is an important electron transfer mediator enabling *P. tannophilus* to reduce heavy metals e.g. Cr(VI). pH is the most influential variable in natural environment. With a decrease of pH, the amounts of released heavy metals and other contaminants increase and result in high toxicity [37]. The results in Fig. 4 are helpful in enabling understanding of fungi-predominated biogeochemical processes in the environment *e.g.*in acidic tea orchard soil.

4. Conclusions

Electrochemical techniques have been employed in studying the riboflavin mediated, extracellular, reduction processes of a model yeast *P. tannophilus*. The experiments indicated that *P. tannophilus* is an electrochemically active species. The EET capacity of *P. tannophilus* is shuttled by riboflavin as the mediator. Moreover, *P. tannophilus* has been shown to secrete riboflavin in a weakly acidic environment; the highest concentration was 0.26 μmol/g biomass at pH 3. This discovery sheds light on an EET mechanism of fungi and could contribute to the environmental application of MFCs for the remediation of acidic pollution sites.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.electacta.2016.05.139.

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